

Impact of Extraction Methods on the Yield and Quality of Essential Oil from Oregano (*Origanum Vulgare*, Subsp. *Vulgare*)

Ema Shahini^{*1}, Ela Hoti², Ledjan Malaj³, Shpend Shahini⁴

¹Department of Pharmacy, University of Medicine Tirana, Tirana, Albania.

Email: emashahini030@gmail.com | ORCID: <https://orcid.org/0009-0001-2119-054X>

²Department of Pharmacy, University of Medicine Tirana, Tirana, Albania.

Email: ela.hoti312@outlook.com | ORCID: <https://orcid.org/0000-0003-1193-5357>

³Department of Pharmacy, University of Medicine Tirana, Tirana, Albania.

Email: l-malaj3@hotmail.com | ORCID: <https://orcid.org/0000-0001-9916-9858>

⁴Department of Plant Protection, Agricultural University of Tirana, Tirana, Albania.

Email: s_shahini@outlook.com | ORCID: <https://orcid.org/0009-0009-6885-0179>

**Corresponding author*

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Abstract

The research investigated the effects of different extraction methods on the yield and chemical composition of essential oil obtained from the flowers, leaves, and stems of *Origanum vulgare* subsp. *Vulgare* (Oregano). The study employed hydrodistillation, Soxhlet extraction, and ultrasonic extraction using n-hexane and 70% ethanol as solvents. Ultrasonic extraction with n-hexane yielded the highest amount of essential oil (1.35% of sample weight), while hydrodistillation with toluene produced the lowest yield (0.25%) from stem samples. Chemical composition analysis revealed sabinene as the dominant component of the essential oil, with its concentration varying based on the extraction method and solvent used, reaching 100% with ultrasonic extraction using 70% ethanol. In stem samples, terpinen-4-ol (20.3%) was notably abundant following hydrodistillation, while in the flowers and leaves sample group, the highest terpinene content (34.3%) was observed using the Soxhlet method. The results underscore the importance of optimising extraction methods and solvent selection to maximise yield while preserving the qualitative composition of essential oils. The above data confirm that ultrasonic extraction is superior in terms of both efficiency and preservation of the chemical composition. Future research should focus on optimising ultrasonic extraction parameters, including solvent type and temperature, to further enhance the yield of key essential oil components while preserving their quality.

Keywords

Hydrodistillation; Plants; Gas chromatography; Phytochemistry; Antioxidant power; Morphological parts of plants

Introduction

The essential oil of *Origanum vulgare* (commonly referred to as Oregano) possesses strong antioxidant, antimicrobial and anti-inflammatory properties, rendering it a valuable product widely

utilised in the pharmaceutical, food, and cosmetics industries. Factors such as extraction methods, growing conditions, and solvent selection play a key role in determining the yield and chemical composition of the oil. Previous studies have investigated various aspects of essential oil extraction techniques from *Origanum vulgare* and their influence on the quality of the final product. For instance, Caputo *et al.* (2022) demonstrated that drying methods play a pivotal role in determining the content of key components, such as sabinene and carvacrol. The authors emphasised the importance of adequately preparing plant materials before extraction to optimise the preservation of the chemical structure of bioactive compounds.

Węglarz *et al.* (2020) examined the effects of Central European climatic conditions on the quality of the essential oil derived from *Origanum vulgare*. Their findings revealed that variations in cultivation temperature have a significant impact on the levels of antioxidants and terpenoids, which are essential for pharmaceutical and food applications. Innovative extraction methods, such as ultrasonic extraction, were investigated by Hrnčič *et al.* (2020). The study compared ultrasonic extraction with those of conventional methods such as hydrodistillation and Soxhlet extraction. Based on the findings, it was concluded that ultrasound offers significant advantages in preserving the oil's qualitative composition. However, further standardisation of ultrasonic extraction conditions is needed to ensure consistent and reproducible results.

A study conducted by Ilić *et al.* (2022) investigated the impact of different morphological parts of the plant (flowers, leaves, and stems) on the composition of the essential oil. Their findings revealed significant variations in component concentrations depending on the plant organ, highlighting the need for further detailed studies to optimise raw material collection and processing. Additionally, Lombrea *et al.* (2020) further delved into the phytochemistry of *Origanum vulgare*, emphasising the critical role of solvent selection in determining the quantity and quality of bioactive compounds, such as phenols and terpenes, responsible for the oil's antimicrobial activity. Investigative efforts of Kosakowska, Węglarz and Bączek. (2019) studied the yield and quality of essential oil from *Origanum vulgare* subsp. *hirtum*, grown organically in a temperate climate. Study results demonstrated that climatic conditions and organic farming practices significantly influence the oil's antioxidant and antimicrobial components, especially carvacrol. Meanwhile, Wu *et al.* (2019) introduced an innovative application of the vacuum distillation technique for essential oil extraction, showcasing significant advantages, preserving sensitive components and improving process efficiency.

The effect of harvest time on the quality and quantity of essential oil from *Origanum vulgare* subsp. *gracile* was examined by Moradi *et al.* (2021). The authors concluded that late harvest increases the concentration of terpenoids such as thymol and sabinene, although precise harvest timeframe optimisation remains undetermined. Jan *et al.* (2020) conducted a comparative analysis of the bioactivity of oil derived from cultivated and wild ecotypes of *Origanum vulgare*, indicating that wild ecotypes possess higher antimicrobial potential, attributed to higher concentration of phenolic compounds. Žitek *et al.* (2021) optimised methods for extracting phenolic compounds with antimicrobial properties from *Origanum vulgare* by carefully choosing the solvent and extraction conditions, as even slight adjustments in temperature or pressure can significantly impact the composition of the extracted compounds.

Although several advancements have been made, as demonstrated above, the challenge of optimising extraction methods to achieve the highest yield of essential oil with the most desirable chemical profile persists. Future essential investigative studies include the impact of solvents on the chemical composition, the relationship between extraction time and essential oil yield, and the dependence of oil quality on the specific plant organs used.

This study aims to conduct a comparative analysis of four extraction methods – hydrodistillation, Soxhlet method, and ultrasonic extraction using n-hexane and 70% ethanol – for *Origanum vulgare* subsp. *vulgare*, to identify the optimal method to achieve the highest oil yield and a high concentration of target components (sabinene and terpinen-4-ol). The objectives of this study are: (1) to investigate the influence of different extraction methods and temperature variations (35°C and 55°C) on the quantitative yield and qualitative composition of *Origanum vulgare* essential oil; (2) to determine the chemical composition of the essential oil from various morphological parts of the plant (flowers, leaves, and stems); and (3) to identify the optimal solvent for ultrasonic extraction that maximizes efficiency while preserving bioactive compounds.

Materials and Methods

Collection of plant material: *Origanum vulgare* subsp. *vulgare*. The plant material used in this study was obtained from the collection of the Institute of Plant Genetic Resources in Albania, a leading institution for the study and conservation of genetic plant resources in the region. The collected sample is derived from red oregano (*Origanum vulgare* subsp. *vulgare*). The following flow chart outlines the systematic research procedure employed to compare different extraction methods for obtaining essential oil from *Origanum vulgare* subsp. *vulgare* (Figure 1).

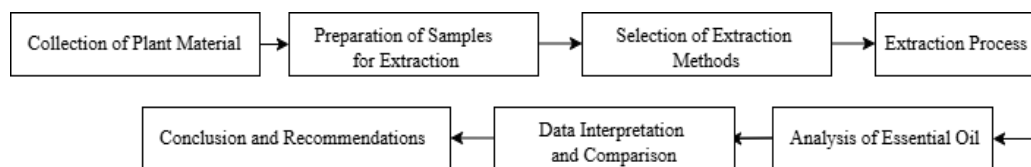


Figure 1: Research procedure for extracting essential oil from *Origanum vulgare* subsp. *vulgare*

The material was harvested during the full flowering period (June to September 2024), which is known to yield the highest concentration of volatile compounds, essential for obtaining high-quality oil. After collection, the plant material was stored in paper packages in a cool, dark environment, protected from air humidity. Depending on the extraction method, the plant material was divided into two categories: mixtures of flowers and leaves, and stems. The essential oil was initially extracted using the hydrodistillation method with a Clevenger-type apparatus, following the protocol outlined in the European Pharmacopoeia. A 100 g sample of dry, crushed plant material (flowers and leaves) was placed in a 1000 ml round-bottom flask, to which 500 ml of distilled water was added. The distillation process was conducted for three hours at a flow rate of 2-3 ml/min. A graduated tube was utilised to obtain the volume of the essential oil, and the yield was calculated as a percentage of the dry material weight (volume/weight). The extracted essential oil was contained in glass vials and optimally

stored at 4°C with acceptable temperature variations between 2-8°C, awaiting further analysis by gas chromatography (GC).

Solvent hydrodistillation was conducted by utilising a Clevenger-type apparatus. The dry, crushed plant material was divided into two samples: one consisting of a mixture of flowers and leaves, and the other of stems. Each sample (100 g) was placed in a 1,000 ml flask with 500 ml of distilled water, along with 2 ml of toluene as a solvent. Toluene application in a modified hydrodistillation protocol led to a slight increase in essential oil yield from the flower-leaf mixture (0.82% vs. 0.8%) and significantly altered the chemical profile, enhancing the recovery of components like sabinene and terpinen-4-ol. The procedure was carried out for three hours. The essential oil obtained was stored in sealed bottles and stored at 4°C until further analysis.

Extraction using a Soxhlet apparatus was conducted as follows: the dry, crushed material was divided into two samples: a mixture of flowers and leaves, and stems. Each sample (10 g) was placed in the filter cartridge of the apparatus. The apparatus was connected to a condenser and a 300 ml flask. Petroleum ether, with a boiling point of 40-60°C, was used as the solvent. The procedure lasted for 8 hours at a rate of 6-7 cycles per hour. After the extraction, the solvent was evaporated, the flask dried to a constant weight, and the yield of essential oil was calculated as a percentage (volume/weight) of the dry mass. The ultrasonic extraction method was performed using an ultrasonic water bath. Two samples of the mixture of flowers and leaves (2 g each) were placed in dried containers, and 30 ml of solvent (n-hexane or 70% ethanol) was added. Ethanol served as a compromise solvent, improving the extraction of essential oil components by enhancing solubility through hydrogen bonding and improving mass transfer under ultrasonic conditions. In addition, its miscibility with water allows selective extraction of bioactive compounds while maintaining a safer and more environmentally friendly profile compared to purely non-polar solvents. The extraction was conducted at a temperature of 35°C for 25 minutes. After extraction, the solutions were filtered, the solvents evaporated, and the containers dried to a constant weight. The obtained essential oil samples were stored at a temperature of 4°C.

For the gas chromatography phase, essential oils from seven samples were analysed using a Varian 450 GC gas chromatograph (USA) equipped with a flame ionisation detector (GC/FID). Each sample (1 µl) was introduced into a VF-1ms capillary column (30 m×0.33 mm×0.25 µm). Nitrogen was used as the carrier gas at a flow rate of 1 ml/min. The temperature regime was as follows: 40°C (2 min), 150°C (4°C/min), 280°C (10°C/min, 2 min). For a comprehensive evaluation of the results, chemical components were identified using Kovats indices, the National Institute of Standards and Technology database (2023), and literature data (Adams, 2017).

A limitation of the study is that it investigates only a narrow range of solvents and excludes root samples, which may affect the generalizability of the findings to other extraction contexts. Additionally, the study does not account for seasonal variations in oil composition, which could provide valuable insights into how climatic and temporal factors, such as temperature and humidity fluctuations, influence both the yield and quality of essential oils over time. These factors could significantly impact the chemical profile of the oils and warrant further investigation for a more comprehensive understanding.

Results and Discussion

The analysis of essential oil yields obtained through different extraction methods reveals that the percentage yield varies depending on both the extraction technique and the plant material used (Table 1). The highest yield of essential oil – 1.35% – was achieved via ultrasonic extraction using n-hexane as a solvent for a 2 g sample. In contrast, the lowest yield, 0.25%, was observed with hydrodistillation of the stem sample (100 g) using toluene as the solvent. A comparison of the methods shows that hydrodistillation requires the largest sample volume (100 g), whereas only 10 g is needed for extraction using the Soxhlet apparatus, and as little as 2 g for ultrasonic extraction. The analysis also indicates that hydrodistillation without solvents produced a yield of 0.8%, while the addition of toluene resulted in varying yields: 0.82% for the flower-leaf mixture and 0.25% for the stem sample. The Soxhlet method demonstrated a yield of 1.3% for the flower-leaf mixture and 0.3% for the stems. The ultrasonic method, applied to the same samples, yielded 1% with 70% ethanol and 1.35% with n-hexane.

Table 1: Percentage (volume/weight) of essential oil obtained by extraction methods

Attribute	Hydrodistillation (Flowers and leaves) Solvent free	Hydrodistillation (Flowers and leaves) Solvent: toluene	Hydrodistillation (stem) Solvent: toluene	Extraction with Soxhlet apparatus (flowers and leaves)	Extraction with the Soxhlet apparatus (stem)	Ultrasonic extraction (Flowers and leaves) Solvent: ethanol 70%	Ultrasonic extraction (Flowers and leaves) Solvent: n-Hexane
Sample amount	100 g	100 g	100 g	10 g	10 g	2 g	2 g
Extraction time	3 hours	3 hours	3 hours	8 hours	8 hours	25 minutes	25 minutes
Extraction temperature	100°C	100°C	100°C	40-60° C	40-60° C	35°C	35°C
Amount of essential oil obtained	0.8 g	0.82 g	0.25 g	0.13 g	0.03 g	0.02 g	0.027 g
The percentage of essential oil obtained from the sample	0.8%	0.82%	0.25%	1.3%	0.3%	1%	1.35%
The colour of the obtained essential oil	Yellow	Yellow	Light yellow	Yellow to green	Light yellow	Light yellow	Yellow to brown

Thus, the method requiring the largest sample volume (hydrodistillation) yielded the lowest result (0.25% from stems), while the method using the smallest sample volume (ultrasonic, 2 g) achieved the highest yield (1.35% from a mixture of flowers and leaves). The extraction time varied significantly: 25 minutes for ultrasonic, 3 hours for hydrodistillation, and up to 8 hours for the Soxhlet method. The extraction temperatures ranged from 35°C (ultrasonic), 40-60°C (Soxhlet), to 100°C (hydrodistillation). The colour of the essential oils varied depending on the extraction method. For instance, during stem sample extractions, hydrodistillation with toluene produced a light yellow oil, whereas ultrasonic extraction with n-hexane resulted in a rich yellow-brown oil. The

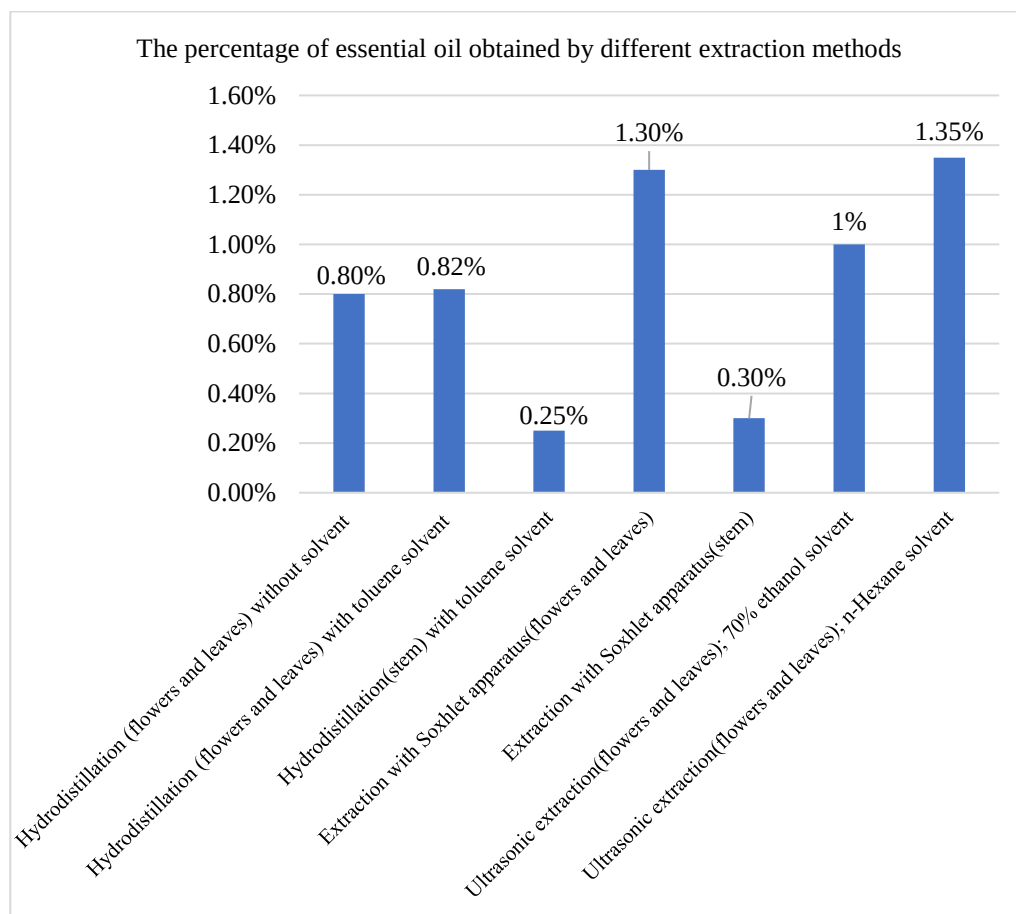
high solubility and effective extraction capabilities of the solvent with plant materials enhance its ability to break down the cell structure and release essential oil components efficiently. Table 1 summarises the data for each extraction method, detailing sample type, sample amount, extraction time, temperature, essential oil yield, yield percentage, and colour. Notably, for the ultrasonic method applied to the same sample (2 g of flowers and leaves), the choice of solvent yielded different results: 1.35% with n-hexane and 1% with 70% ethanol. This highlights the significant impact of solvent type on the extraction outcome.

The results of the two traditional extraction methods (Clevenger hydrodistillation and Soxhlet extraction) show significant differences in the sample quantities used (100 g vs. 10 g) and the yield of essential oil. The maximum percentage yield for hydrodistillation is 0.82% (from the flower and leaf sample using toluene), while the Soxhlet method achieves a higher yield of 1.3% for the same sample type. The increased yield in the Soxhlet method can be attributed to its prolonged extraction time of 8 hours. Lower yields were observed for the stem samples: 0.25% with hydrodistillation using toluene and 0.3% with the Soxhlet method using petroleum ether as the solvent. This confirms that the essential oil content in the stems is lower than in the flowers and leaves. Even for stems, the Soxhlet method demonstrates a higher yield compared to hydrodistillation.

Comparison of methods for the same sample type

For both methods, the maximum percentage yield was observed in the flower and leaf samples compared to the stem samples. For instance, in the Soxhlet method, the yield was 1.3% for flowers and leaves, but only 0.3% for stems. Similarly, in hydrodistillation, the yield for flowers and leaves (with toluene) was 0.82%, compared to 0.25% for stems. Even within the same method (hydrodistillation), the use of a solvent slightly increased the yield. The yield for flowers and leaves without solvent was 0.8%, while with toluene it increased to 0.82%. This indicates that toluene positively impacts essential oil yield, even when extracting from the same plant organ (flowers and leaves). However, the variation in yield depends not only on the extraction method but also on the specific plant organ used (Allaberdiev *et al.*, 2021; Zubtsova and Skliar, 2023). The choice of solvent significantly affected essential oil yield: among the tested solvents (toluene, petroleum ether, 70% ethanol, and n-hexane), n-hexane produced the highest yield (1.35%) in flower and leaf samples, while toluene resulted in the lowest yield (0.82%). In stem samples extracted using traditional methods, toluene resulted in the lowest essential oil yield (0.25%), while petroleum ether yielded slightly more at 0.3% (Figure 2).

The yield of essential oil varies significantly depending on the extraction method and the solvent used. This variability highlights the critical importance of optimising extraction conditions to achieve maximum efficiency. To assess the chemical composition of the essential oil obtained from *Origanum vulgare* subs. *vulgare*, gas chromatography with a flame ionisation detector (GC/FID) was used to analyse 23 major chemical components. The results are presented as percentage values for individual samples, which include a flower-leaf mixture and stem samples. Table 2 summarises the chemical composition of the flower-leaf mixture samples extracted using the methods studied.

Figure 2: Data on essential oil *Origanum vulgare* subs. *Vulgare* extraction methodsTable 2: Chemical components of essential oil obtained from a mixture of flowers and leaves by the methods used in the study of *Origanum vulgare* subsp. *vulgare*

Chemical components	Hydrodistillation Solvent free	Hydrodistillation with toluene solvent	Extraction with the Soxhlet apparatus	Ultrasonic extraction Solvent: n- Hexane	Ultrasonic extraction Solvent: ethanol 70%
alpha-Thujene	0.8	1.7	1.5	1	0
alpha-Pinene	0.3	0.8	0.6	0	0
Sabinene	10.3	18	17.3	13.9	100
beta- Pinene	0.5	2.4	2.3	2.1	0
Myrcene	1.6	4.4	4.2	1.4	0
alpha-Phellandrene	0.1	0	0	-	0
Alpha- Terpinene	2.6	8.6	5.6	3.7	0
p-Cymene	-	3.5	3.9	0	0
Limonene	3.2	4.7	6.3	0	0
Beta- Phellandrene	2.7	3.1	4.6	0	0
gamma- Terpinene	4.3	1	0.7	0	0
Cis-Sabinene Hydrate	2.9	2.9	3.4	4.2	0

Terpinolene	2.2	2.1	1	0	0
Linalool	0.7	3.9	2.8	4.8	0
Trans- Sabinene Hydrate	4.7	9.1	6.9	7.8	0
Cis-p-menthen-2-ol	0.8	1.1	1.2	5.3	0
Trans-p-menthen-2-ol	0.3	0.7	0	2.2	0
Borneol	1	0	0	1.7	0
Terpinen-4-ol	4.9	9	13.5	19.9	0
alpha-Terpineol	4.1	1.1	13.5	12.4	0
Linalyl acetate	1.1	2.6	1.7	1.5	0
Thymol	1.1	1	0	2.1	0
Carvacrol	1.5	0.5	0	0	0
Total (%)	51.7	82.2	91	84	100

Chemical composition of essential oil in flower and leaf mixture samples depending on extraction methods

The chemical composition of essential oil extracted from flower and leaf mixtures varied notably with the extraction method. The total content of the 23 primary components ranged from 51.7% with solvent-free hydrodistillation, 82.2% with hydrodistillation using toluene, 84% with ultrasonic extraction using n-hexane, and 91% with Soxhlet extraction, to 100% with ultrasonic extraction using 70% ethanol. Sabinene is identified as the primary component in the essential oil across all flower and leaf samples. Its percentage content shows notable differences based on the extraction method: 17.9% in solvent-free hydrodistillation, 25.9% in ultrasonic extraction with n-hexane, 27.6% in the Soxhlet method, 30% in hydrodistillation with toluene, and 100% in ultrasonic extraction with 70% ethanol. Figure 3 shows the results of the percentage of total sabinene in the essential oil for samples from a mixture of flowers and leaves using different extraction methods. It is also observed that the content of total sabinene can vary even within the same extraction method, depending on the solvent used (e.g., toluene in hydrodistillation). Similarly, the effect of the solvent is noticeable when the same method (ultrasonic extraction) is used with different solvents.

Results of the study of the chemical composition of the essential oil. In the samples of the mixture of flowers and leaves, variations in the percentage of total terpenes were found depending on the extraction method. The values vary as follows: 18.1% (hydrodistillation without solvent), 21.8% (hydrodistillation with toluene), 34.3% (Soxhlet method), 36% (ultrasonic extraction with n-hexane). When using ultrasonic extraction with 70% ethanol, the presence of terpinene was not recorded in the composition of the essential oil (Figure 4).

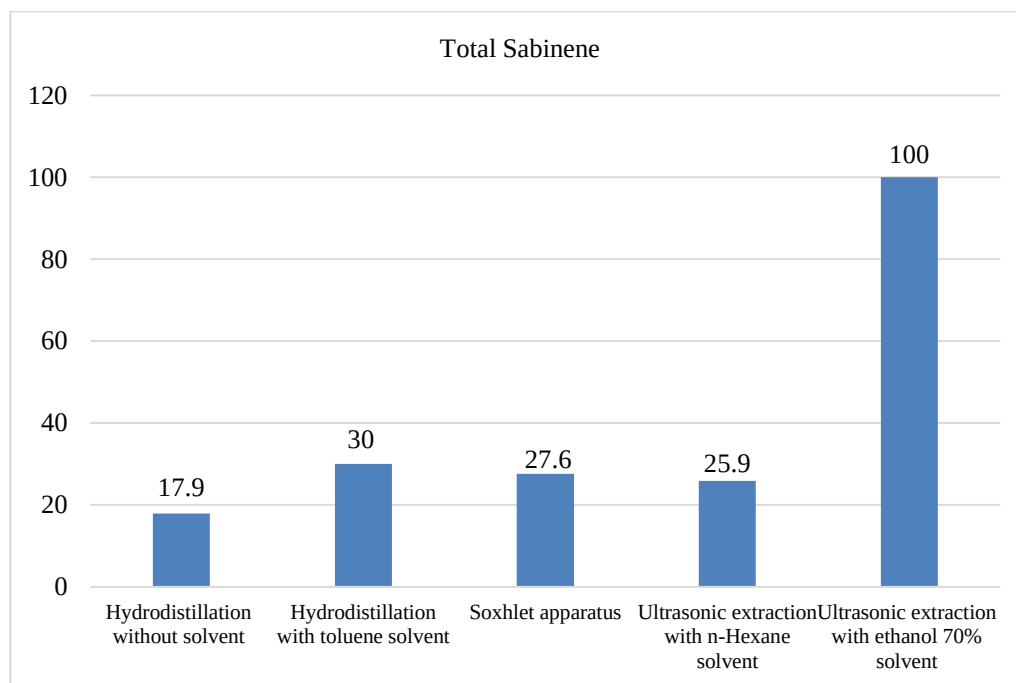


Figure 3: Percentage of total sabinene in essential oil in flower and leaf mixture samples depending on extraction methods

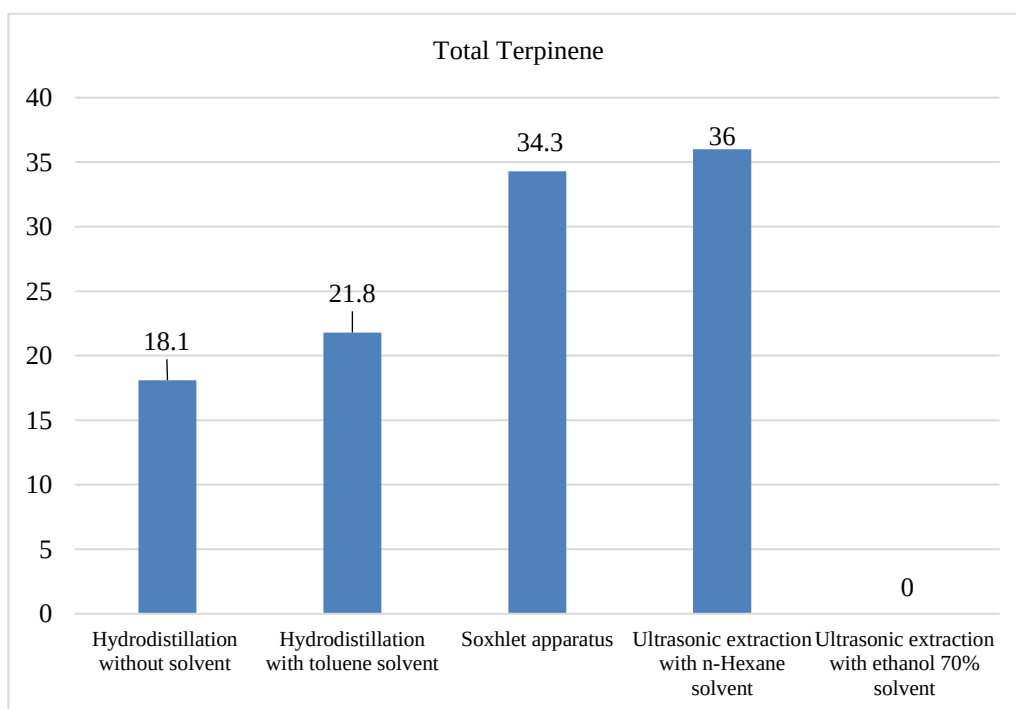


Figure 4: Percentage of total terpenes in flower and leaf mixture samples depending on extraction methods

Similar to sabinene, the total terpene content varies significantly across different extraction methods and also within the same method, depending on the solvent used (e.g., hydrodistillation or ultrasonic extraction). The results confirm that solvent choice significantly affects the qualitative and quantitative composition of the obtained essential oil. In stem samples, chemical variability is also observed depending on the extraction method. For instance, during hydrodistillation with toluene, the compounds with the highest percentage values include: terpinen-4-ol (20.3%), trans-sabinene hydrate (14%), alpha-terpineol (12.6%), while sabinene is present in much lower concentrations (1.5%). In the Soxhlet method, on the other hand, sabinene occupies a dominant position, reaching 100%. Table 3 shows the chemical components in stem samples based on the methods used in the extraction study.

Table 3: Chemical components of essential oil extracted from stem samples using the studied methods

<i>Chemical components</i>	<i>Hydrodistillation with toluene solvent</i>	<i>Extraction with the Soxhlet apparatus</i>
alpha-Thujene	1.7	0
alpha-Pinene	0.6	0
Sabinene	1.5	100
beta- Pinene	0	0
Myrcene	0.7	0
alpha-Phellandrene	0	0
Alpha- Terpinene	7.4	0
p- Cymene	1.7	0
Limonene	5.6	0
Beta- Phellandrene	2.6	0
gamma-Terpinene	-	0
Cis-Sabinene Hydrate	3.6	0
Terpinolene	-	0
Linalool	2.8	0
Trans- Sabinene Hydrate	14	0
Cis-p-menthen-2-ol	1.3	0
Trans-p-menthen-2-ol	2.3	0
Borneol	5.8	0
Terpinen-4-ol	20.3	0
alpha-Terpineol	12.6	0
Linalyl acetate	2.5	0
Thymol	0	0
Carvacrol	0	0
Total (%)	87	100

In general, for hydrodistillation, terpinen-4-ol and trans-sabinene hydrate yield the highest percentage, while in the Soxhlet method, they were not detected at all. Some components, such as beta-pinene, alpha-phellandrene, gamma-terpinene, terpinolene,

thymol, and carvacrol, are not recorded in both methods for stem samples. General analysis of the chemical composition of the different samples. All extraction methods show variability in the components depending on the type of sample (flowers, leaves, or stems) (Figure 5). For hydrodistillation using toluene, the most important component in flower and leaf samples is sabinene (30%), while for stems, terpinene dominates (40.3%). In the Soxhlet method, flower and leaf samples show a higher content of terpinene (34.3%), while in stems, sabinene is the prominent component (100%).

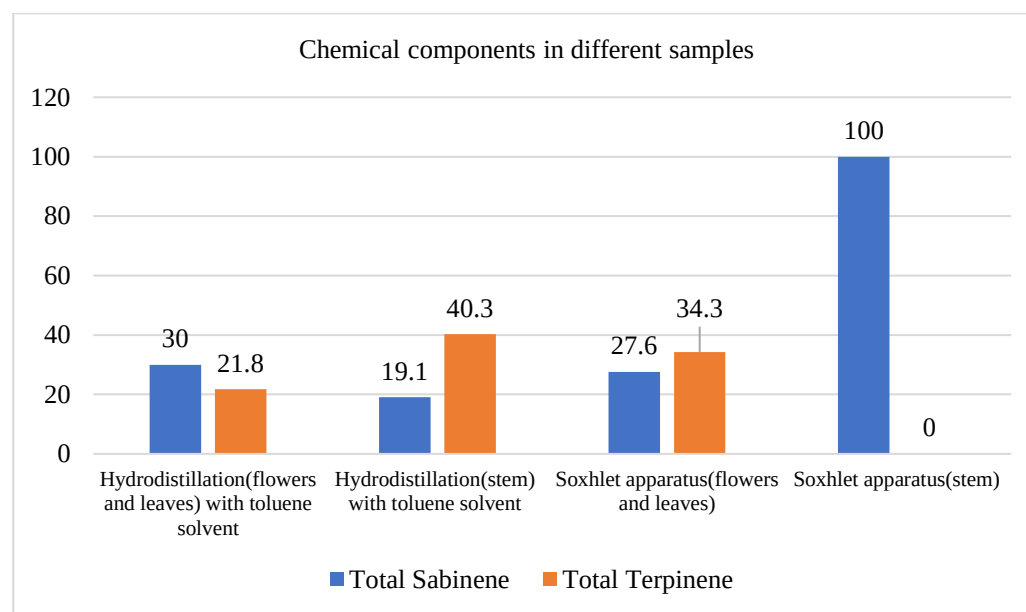


Figure 5: Chemical composition of the main components for different samples using the same extraction method

Table 4 summarises the results and contains a detailed distribution of components for all samples according to the method used. It was shown that the quantity and quality of the essential oil varied significantly depending on the extraction method, the type of solvent, and the morphology of the plant material.

The content of chemical components in different samples varies depending on the extraction method. The total percentage of chemical components ranges from 51.7% (hydrodistillation of a mixture of flowers and leaves without solvent), 82.2% (hydrodistillation with toluene), 84% (ultrasonic with n-hexane), 87% (hydrodistillation of a stem sample with toluene), and 91% (Soxhlet extraction for flowers and leaves) to 100% (for stems in the Soxhlet method and ultrasonic extraction of a mixture of flowers and leaves with 70% ethanol). The highest values in these samples are provided by sabinene. During hydrodistillation, individual components such as alpha-thujene are present in small percentages (from 0.8% to 1.7%), with the second value being the same for the two samples with toluene. Alpha-pinene (0.3% to 0.8%) and cis-p-menthen-2-ol (0.8% to 1.3%) are also detected. Thymol and carvacrol are absent in stem samples with toluene; however, for flowers and leaves, these components are present at 1.1% and 1.5%, respectively.

Table 4: Chemical components of essential oil for each sample by extraction methods

Chemical components	Hydro-distillation of flowers and leaves Without solvent	Hydro-distillation flowers and leaves (toluene solvent)	Hydro-distillation stem (toluene solvent)	Extraction with the Soxhlet apparatus of flowers and leaves	Extraction with the Soxhlet apparatus stem	Ultrasonic extraction with n-Hexane solvent	Ultrasonic extraction with 70% ethanol solvent
alpha-Thujene	0.8	1.7	1.7	1.5	0	1	0
alpha-Pinene	0.3	0.8	0.6	0.6	0	0	0
Sabinene	10.3	18	1.5	17.3	100	13.9	100
beta- Pinene	0.5	2.4	0	2.3	0	2.1	0
Myrcene	1.6	4.4	0.7	4.2	0	1.4	0
alpha-Phellandrene	0.1	0	0	0	0	-	0
Alpha-Terpinene	2.6	8.6	7.4	5.6	0	3.7	0
p- Cymene	-	3.5	1.7	3.9	0	0	0
Limonene	3.2	4.7	5.6	6.3	0	0	0
Beta-Phellandrene	2.7	3.1	2.6	4.6	0	0	0
gamma-Terpinene	4.3	1	-	0.7	0	0	0
Cis-Sabinene Hydrate	2.9	2.9	3.6	3.4	0	4.2	0
Terpinolene	2.2	2.1	-	1	0	0	0
Linalool	0.7	3.9	2.8	2.8	0	4.8	0
Trans-Sabinene Hydrate	4.7	9.1	14	6.9	0	7.8	0
Cis-p-menthen-2-ol	0.8	1.1	1.3	1.2	0	5.3	0
Trans-p-menthen-2-ol	0.3	0.7	2.3	0	0	2.2	0
Borneol	1	0	5.8	0	0	1.7	0
Terpinen-4-ol	4.9	9	20.3	13.5	0	19.9	0
alpha-Terpineol	4.1	1.1	12.6	13.5	0	12.4	0
Linalyl acetate	1.1	2.6	2.5	1.7	0	1.5	0
Thymol	1.1	1	0	0	0	2.1	0
Carvacrol	1.5	0.5	0	0	0	0	0
TOTAL (%)	51.7	82.2	87	91	100	84	100

It should be noted that the stability of essential oils, including those of *Origanum vulgare subsp. vulgare*, during long-term storage, is highly dependent on multiple factors such as storage temperature, light and container type. In the present study, essential oil samples after extraction were stored in airtight glass vials at 4°C with acceptable temperature fluctuations between 2-8°C to maintain their chemical integrity until analysis. Although no specific long-term stability experiments were performed as part of this study, it is well known that low temperatures and protection from light and humidity minimise oxidative degradation and volatilisation of key components such as sabinene and terpinen-4-ol. The absence of light and limited exposure to oxygen retards peroxide formation and loss of volatile terpenoids, allowing essential oils to retain their qualitative and quantitative characteristics over time.

The absence or minimal presence of thymol and carvacrol in the essential oil samples extracted in this study, despite their well-documented prevalence in oregano oil, is attributed to multiple interrelated factors, including the specific subspecies used (*Origanum vulgare* subsp. *vulgare*), the choice of extraction method, and solvent polarity. Unlike *Origanum vulgare* subsp. *hirtum*, which is typically richer in thymol and carvacrol, the subspecies examined here is known to exhibit a chemotype with a higher concentration of sabinene and other monoterpenes, thereby inherently producing oil with a different phytochemical profile. Additionally, the use of ultrasonic extraction with non-polar or mildly polar solvents (such as n-hexane and 70% ethanol) under relatively low temperatures (35°C) may have contributed to the limited extraction of thymol and carvacrol, which are phenolic compounds with higher polarity and a tendency to degrade or remain bound in more complex plant matrices. Hydrodistillation, while typically effective in recovering phenolics, was performed under standard conditions that may not have been optimised for thymol/carvacrol liberation from this particular plant tissue, especially stems, which generally possess lower essential oil content and reduced concentrations of phenolic compounds.

The Soxhlet method demonstrates a high total content of chemical components (91%-100%), but their percentages differ significantly between samples. Sabinene is the dominant component: 17.3% in flower-leaf samples and 100% in stem samples. Meanwhile, components such as alpha-phellandrene, trans-p-menthen-2-ol, borneol, thymol, and carvacrol are not detected in this method. In ultrasonic extraction, the percentage composition of the components varies significantly depending on the solvent used. With n-hexane, terpinen-4-ol shows the highest value (19.9%), while with 70% ethanol, sabinene (100%) predominates. Some components, including alpha-pinene, alpha-phellandrene, para-cymene, limonene, beta-phellandrene, gamma-terpinene, terpinolene, and carvacrol, are almost undetectable. Across all extraction methods, alpha-phellandrene shows the lowest content, 0.1% in solvent-free hydrodistillation and 0% in the other methods. In contrast, sabinene exhibits the highest values, ranging from 13.9% to 100%, confirming its dominant role among the essential oil components depending on the extraction conditions.

The superiority of n-hexane over ethanol in ultrasonic extraction can be attributed to differences in solvent polarity and solvation. n-Hexane is a non-polar solvent that effectively dissolves non-polar compounds such as monoterpenes and sesquiterpenes, key components of essential oils such as sabinene and terpinen-4-ol. Its non-polar nature promotes deeper penetration into the plant matrix and enhances the cavitation effect under ultrasonic conditions, resulting in better rupture of plant cell walls and release of hydrophobic bioactive compounds. In contrast, 70% ethanol, being a polar protic solvent, preferentially extracts polar compounds and is less effective in solubilising non-polar volatile components predominant in oregano oil. This polarity mismatch reduces the overall yield of essential oil, as evidenced by the lower extract yield (1.0%) compared to n-hexane (1.35%). Moreover, although ethanol afforded a composition rich in sabinin, its selectivity was at the expense of other terpenoids such as terpinenes, which were practically absent, emphasising that the polar nature of ethanol narrows the spectrum of extractable compounds despite increasing the purity of specific targets.

When evaluating the environmental and economic trade-offs of different essential oil extraction methods, several factors must be considered, particularly energy consumption and solvent costs. Hydrodistillation, although one of the most traditional and widely used methods, is energy-intensive, requiring long heating times and large volumes of water, resulting in higher energy costs and environmental impacts due to the use of non-renewable energy sources. The Soxhlet method, although efficient, is also energy-intensive as it requires a long extraction time and constant use of heated solvents. Ultrasonic extraction, on the other hand, offers significant energy savings due to shorter extraction times and lower temperature requirements, making it more energy efficient.

However, the economic viability of this method depends on the cost of solvents: non-polar solvents such as n-hexane are relatively inexpensive, but their environmental impact can be significant due to their toxicity and inability to biodegrade. The use of 70% ethanol, although more environmentally friendly and safer to handle, increases operating costs due to its higher price compared to non-polar solvents. Thus, while ultrasonic extraction using ethanol may be environmentally preferable, the higher cost of the solvent may limit its economic feasibility, especially in large-scale commercial operations. Ultimately, a balance between extraction efficiency, solvent selection, energy consumption, and environmental sustainability must be struck to optimise economic and environmental outcomes.

Environmental factors such as temperature, humidity and light play an important role in shaping the chemical composition and yield of essential oils. Temperature affects the rate of essential oil synthesis in plants: extreme heat can lead to degradation of volatile compounds, while cooler conditions can slow down the production of biologically active components. Humidity, on the other hand, can affect the moisture content of plant materials, which affects the efficiency of extraction processes and the stability of the oils obtained. High humidity can promote microbial growth, which degrades oil quality, and low humidity can lead to excessive drying of the plant material, which reduces its yield. In addition, exposure to light can accelerate the oxidation of essential oils, leading to the degradation of sensitive compounds such as terpenes. To achieve optimum quality and yield, it is important to control these environmental conditions both during plant cultivation and during storage of the oils after extraction, ensuring that their chemical profile and bioactivity are preserved.

The analysis of the obtained data allows for a comparison of the efficiency of hydrodistillation, ultrasonic extraction, and Soxhlet extraction methods with previous studies, expanding our understanding of how extraction technologies influence the composition and properties of essential oils. The data emphasise the importance of sabinene as a key component of the oil. This finding perfectly aligns with Kosakowska *et al.* (2021), who previously identified sabinene's high antimicrobial potential. The results also show that the ultrasonic method with n-hexane achieves the highest oil yield (1.35%) in the shortest amount of time (25 minutes). The result is further supported by Alekseeva *et al.* (2020), which highlights the advantages of modern extraction technologies in preserving sensitive components. However, the data on the content of sabinene (up to 100% in ultrasonic extraction with 70% ethanol) underline the specific role of the solvent in ensuring maximum efficiency, which supports the findings of de

Falco *et al.* (2013) regarding the influence of growth conditions and solvents on the chemical profile of essential oil.

A comparison with hydrodistillation confirms its reduced efficiency, especially for stem samples, with a yield of only 0.25%. Similar findings were reported by Hashemi *et al.* (2017), who highlighted that classical hydrodistillation methods are less effective for dense plant materials. The Soxhlet method, despite requiring longer processing time (8 hours), demonstrates a high oil yield for flowers and leaves (91%-100%) and is in line with the research of Napoli *et al.* (2020), who emphasised the method's reliability for extracting terpenoids. Notably, even for stem samples, the Soxhlet method shows greater efficiency compared to hydrodistillation, highlighting its versatility (Havrysh, Nitsenko and Hruban, 2022; Kairbayeva *et al.*, 2022; Uikassova *et al.*, 2022).

Data on the chemical composition of the oil confirm its dependence on the morphological part of the plant, corroborating the conclusions of Baranauskienė *et al.* (2013), who observed that the time of harvest and plant organ significantly influence the concentrations of carvacrol and thymol. Furthermore, the analysis indicates that ultrasonic extraction is optimal for isolating terpinen-4-ol and sabinene, complementing prior findings. Karakaya *et al.* (2011) demonstrated that the choice of extraction method significantly affects the antioxidant and antimicrobial activity of essential oils. The ultrasonic method exhibited high efficiency in preserving key components like carvacrol and thymol, consistent with results supporting the advantages of ultrasonic extraction for maintaining bioactive substances (Coutinho *et al.*, 2016; Sydykova *et al.*, 2023).

The impact of processing methods, including solvent selection, on the final oil composition corresponds with the findings of Tibaldi, Fontana and Nicola (2011). Their study highlights the critical role of cultivation conditions and post-harvest management in determining the chemical composition of essential oils. Specifically, temperature regimes and drying methods significantly influence terpenoid and antioxidant content (Maharramova, 2021; Nabiyeu *et al.*, 2024). Similarly, Özer *et al.* (2018) investigated how drying methods and plant development stages affect oil composition. Their findings reveal that the chemical profile, particularly the content of sabinene and carvacrol, improves under optimal drying conditions, further reinforcing the importance of proper raw material preparation. The antimicrobial and antioxidant properties of the essential oil were found to depend significantly on the extraction method. The findings of Oniga *et al.* (2018), who studied the chemical composition and biological properties of *Origanum vulgare* essential oil, highlight the advantages of modern technologies such as ultrasound, aligning with the conclusions of this study.

Vladić *et al.* (2021) proposed combining enzymatic and microwave raw material preparation with supercritical carbon dioxide extraction to enhance process efficiency. Their results showed significant improvements in the quality and quantity of the oil, supporting the importance of optimising technological parameters. Similarly, Mechergui *et al.* (2016) investigated the impact of the harvest year on the chemical composition and antioxidant activity of the essential oil. It was determined that climatic variations between seasons have a significant impact on terpenoid content, emphasising the necessity for additional research on the seasonal effects on chemical composition. The research by Goncariuc *et al.* (2021) confirmed that stress conditions, such as drought,

substantially affect the quality of *Origanum vulgare* essential oil. Under such conditions, carvacrol and thymol – key compounds with known antimicrobial properties – accumulate in higher concentrations, making the oil particularly promising for pharmaceutical applications (Hussen *et al.*, 2024; Skliar *et al.*, 2023). However, these stress factors also lead to a decrease in total oil yield, aligning with findings on the influence of plant morphology and extraction method. For instance, stem samples exhibited lower yields and a reduced concentration of volatile compounds as compared to flower and leaf samples (Sytnik *et al.*, 2023). This key difference is likely attributable to the structural density of the stems, hindering efficient oil extraction.

The study by d'Antuono *et al.* (2000) emphasises the influence of geographical and climatic factors on the chemical composition of essential oil. The genetic variability of *Origanum vulgare* populations in the Mediterranean region demonstrates that the cultivation site is a critical factor in planning the commercial production of essential oil (Shumka *et al.*, 2021; Yaroshenko, Skliar and Rosenthal, 2023). These findings align with this study's conclusion on the importance of optimising extraction conditions to enhance efficiency. For instance, ultrasonic extraction enables the accommodation of such variables, ensuring a consistently high yield of volatile compounds (Andriievskiy *et al.*, 2024). The research by Majeed *et al.* (2015) significantly advances the understanding of extraction process parameters. The optimisation of temperature, duration, and solvent selection described in their work corroborates the results obtained for ultrasonic extraction, which demonstrated the highest yields of sabinene and terpinen-4-ol. Notably, the use of n-hexane enhances the solubility of monoterpenes, making this method particularly effective for flower and leaf samples.

The findings of Béjaoui *et al.* (2013) on the impact of the plant's phenological stages complement the results of this study. More specifically, their observation that thymol accumulation peaks during flowering aligns with the higher yield and greater chemical richness of essential oil extracted from flower-leaf samples. This highlights the importance of selecting the optimal harvest time to maximise product quality (Zubtsova *et al.*, 2019). A comparison of this study with Béjaoui *et al.*'s findings also underscores the necessity of standardising processing conditions. Although the Soxhlet method is time-intensive, it consistently delivers a high oil yield, making it a strong candidate for industrial applications (Bliznjuk *et al.*, 2022; Korchak *et al.*, 2022). Conversely, ultrasonic extraction stands out for its speed and ability to produce high-quality oil, rendering it highly suitable for pharmaceutical and food applications (Khanzharov *et al.*, 2018).

Novák *et al.* (2011) demonstrated that the drying method significantly impacts the composition of essential oil. Specifically, microwave drying was shown to preserve the highest concentration of volatile compounds, such as carvacrol. This finding underscores the critical importance of proper raw material preparation before extraction (Veliev *et al.*, 2018), as also highlighted in this study. Additionally, Morshedloo *et al.* (2016) investigated the effect of water stress on the chemical composition of essential oil. Their results revealed that stress conditions enhance the synthesis of monoterpenes, such as thymol and carvacrol, aligning with this study's findings and reinforcing the role of external conditions in determining oil quality. The work of Al-Hijazeen *et al.* (2016), which examined the impact of *Origanum vulgare* essential oil on the stability of chicken meat during storage, supports the importance of antioxidants like carvacrol in improving

oxidation resistance. These results are consistent with the data showing that the ultrasonic extraction method yields high-quality oil with potent antioxidant properties. Furthermore, Azizi, Yan and Honermeier (2008) studied the effects of soil moisture levels and nitrogen nutrition on the composition and yield of essential oil. Their findings confirmed that optimal moisture levels promote increased oil yield, though the composition varied with nitrogen levels. This aligns with this study's conclusion that environmental conditions significantly influence the quantitative and qualitative characteristics of essential oil.

Overall, the results of this research are consistent with studies conducted by researchers worldwide. The data collectively emphasise that factors such as environmental conditions, extraction methods, and plant organ types play a decisive role in determining the quality and quantity of essential oils. These findings highlight the critical need for further research to standardise cultivation and extraction practices, ensuring stable and reproducible results.

Conclusion

The results of the study confirmed the significant influence of extraction methods and solvent choice on the yield and quality of essential oil from *Origanum vulgare subsp. vulgare*. A comparative analysis of four methods – hydrodistillation, Soxhlet extraction, ultrasonic extraction with n-hexane, and ultrasonic extraction with 70% ethanol – revealed key quantitative and qualitative patterns. Ultrasonic extraction with n-hexane achieved the highest essential oil yield (1.35%) using minimal raw material (2 g) in the shortest processing time (25 minutes). This method provided the most comprehensive chemical composition (up to 100%), highlighting the advantages of innovative extraction technologies. In contrast, hydrodistillation with toluene yielded the lowest oil content for stem samples (0.25%), requiring 3 hours and 100 g of raw material, demonstrating its limited efficiency for dense plant materials.

Sabinene was identified as a key component of the essential oil, with its concentration varying from 13.9% to 100%, depending on the extraction method. Ultrasonic extraction with 70% ethanol provided the highest sabinene content, underscoring the effectiveness of this solvent for isolating this compound. The Soxhlet method, although time-intensive (8 hours), delivered a high oil yield (91%-100%) for flower and leaf samples, with a well-balanced composition of terpenoids. Hydrodistillation exhibited variability in the composition of specific components, such as terpinen-4-ol, which reached a maximum of 20.3% in stem samples. Ultrasonic extraction with 70% ethanol proved optimal for obtaining pure sabinene, while extraction with n-hexane excelled in isolating terpinene (19.9%). Stem samples consistently showed lower oil yields, likely effect to their dense tissue structure. For maximum efficiency, ultrasonic extraction with n-hexane is recommended for obtaining a broad range of components, while ultrasonic extraction with 70% ethanol is ideal for isolating sabinene.

One of the key limitations of this study is the absence of biological activity assays to assess the antimicrobial, antioxidant, or anti-inflammatory properties of the essential oils extracted using different methods. While the study provides a detailed analysis of extraction efficiency and chemical composition, it does not explore how the variations

in composition, such as the presence of sabinene, terpinen-4-ol, and other terpenoids, might influence the biological activity of the oils. Biological assays, such as disk diffusion tests or DPPH assays, would have provided critical insights into the practical applications of the extracted oils, particularly in the pharmaceutical, food, or cosmetic industries. Additionally, the study does not evaluate the stability or shelf life of the essential oils under different storage conditions, which is crucial for determining their commercial viability. Nevertheless, the findings demonstrate the potential for optimising essential oil extraction processes, which is critical for applications in the pharmaceutical, cosmetic, and food industries. Future research should focus on expanding the variety of solvents, examining seasonal variations in oil composition, and further assessing the effects of climatic conditions.

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Authors' Declarations and Essential Ethical Compliances

Authors' Contributions (in accordance with ICMJE criteria for authorship)

Contribution	Author 1	Author 2	Author 3	Author 4
Conceived and designed the research or analysis	Yes	Yes	Yes	Yes
Collected the data	Yes	Yes	No	No
Contributed to data analysis & interpretation	No	Yes	No	Yes
Wrote the article/paper	Yes	Yes	Yes	Yes
Critical revision of the article/paper	Yes	No	No	Yes
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